responding chromatographically to 3-pyridylacetic acid. 4. Following oral administration of (−)-cotinine to the dog, 3-pyridylacetic acid was isolated from urine and characterized by analysis, melting point, mixed melting point and as its picric acid salt. 5. A precursor of 3-pyridylacetic acid is on formal grounds γ-(3-pyridyl)-β-oxobutyric acid, derivable from the known metabolite γ-(3-pyridyl)-β-oxo-N-methylbutyramide.

1. Ganz, A., Kelsey, F. B., Geiling, E. M. K., *J. Pharmacol. Exp. Therap.*, 1951, v103, 209.

2. Bennett, D. R., Tedeschi, R. E., Larson, P. S., *Arch. Int. Pharmacodyn.*, 1954, v98, 221.

3. McKennis, H., Jr., Turnbull, L. B., Bowman, E. R., *J. Am. Chem. Soc.*, 1958, v80, 6597.

4. McKennis, H., Jr., Bowman, E. R., Turnbull, L. B., *ibid.*, 1960, v82, 3974.

5. McKennis, H., Jr., Turnbull, L. B., Bowman, E. R., Wada, E., *ibid.*, 1959, v81, 3951.

6. McKennis, H., Jr., Wada, E., Bowman, E. R., Turnbull, L. B., in press.

7. Owen, F. B., Jr., Larson, P. S., *Arch. Int. Pharmacodyn.*, 1958, v115, 402.

8. Turnbull, L. B., Bowman, E. R., McKennis, H., Jr., *Fed. Proc.*, 1960, v19, 268.

9. Malon, R. L., Dean, P., *J. Am. Chem. Soc.*, 1947, v69, 1797.

10. Werle, E., Meyer, D., *Biochem. Z.*, 1950, v321, 221.

11. Ginoulhiac, E., Tenconi, L. T., *Min. Med.*, 1959, v5, 1166.

12. Ratti, G., De Fina, E., *Lancet*, 1959, v2, 917.

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Immunoassay of Insulin Content of Crystalline Glucagon Preparations. (26562)

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Purification and crystallization of glucagon (1) have resulted in a preparation of exceedingly high purity. However, since no specific procedure for inactivation of insulin is included in the purification procedure, a slight insulin contamination is virtually unavoidable. It has therefore been suggested(2) that results obtained in biological experiments with glucagon should be interpreted with caution particularly when large doses of glucagon are used. McNaught(3), by measuring glucose uptake and net gas output by slices of lactating rat mammary gland estimated the insulin content of a glucagon preparation to be about 1.0%. Randle(4) determined glucose uptake by rat diaphragm and found the insulin content of an amorphous glucagon preparation to be 1.2%. One crystalline preparation contained less insulin but another (Lot #258-234 B-54-2) produced an even greater increase in glucose uptake than did the amorphous glucagon at the same concentration. J. R-Candela and R. R-Candela (5) reported that glucagon reduced the effect of insulin on glucose uptake and Snedecor *et al.*(6) found a similar inhibition of insulin-induced glycogen deposition. In contrast, Narahara and Williams(7) reported that glucagon potentiates the action of small doses of insulin on diaphragm. Recently, Lee and associates(8) estimated the insulin content of several crystalline glucagon preparations to range from 0.007% to 0.08% by measurement of $C^{14}O_2$ production from glucose-1-C^{14} in the rat epididymal fat pad preparation.

In view of the uncertain effects of glucagon on the response of tissues to insulin and the marked differences in estimated insulin content of crystalline glucagon preparations reported by various investigators an immunologic assay for insulin in 2 glucagon preparations (lots #258-234 B-54-2 and lot #258-234 B-167-1)* was undertaken, the results of which are recorded herein.

Methods. The immunoassay employed here depends on the ability of insulin in unknown solutions to inhibit, competitively, the

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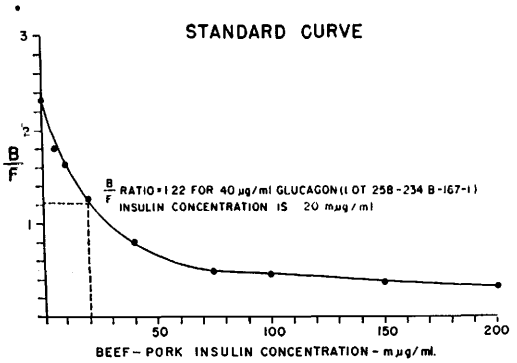

FIG. 1. Ratio of antibody-bound beef-insulin-I^{131} to free insulin-I^{131} (B/F) as a function of concentration of added beef-pork insulin. Ratios were obtained from the complete series of chromatoelectrophoretograms, a few of which are shown on the left side of Fig. 2. The method for determination of insulin content of an unknown sample yielding a B/F ratio of 1:22 is indicated.

combination of I^{131}-labeled insulin with insulin-binding antibodies(9). Degree of reduction in the ratio of antibody-bound insulin-I^{131} (B) to unbound ('free') insulin-I^{181} (F) is a function of the insulin concentration. Separation of bound and free insulin is effected by paper chromatoelectrophoresis wherein the free insulin is adsorbed at site of application(10,11) and antibody-bound insulin migrates with the serum proteins(11).

For preparation of a standard curve, trace amounts of crystalline beef insulin-I^{131} are incubated with known amounts of unlabeled crystalline insulin at various concentrations and with antiserum in fixed concentration. For determination of the insulin content of an unknown solution, an aliquot of the latter is substituted for the insulin employed in the standard solutions. Human serum albumin (2.5 mg/ml) is added to all solutions to prevent losses of insulin by adsorption to glassware. After 4 days at 4°C the mixtures are applied to Whatman 3 MM paper strips for chromatoelectrophoresis(11). The strips are scanned for radioactivity with an automatic recorder and the areas under the bound and free peaks determined with a planimeter. The B/F ratios in standard solutions are plotted against the known insulin concentrations (Fig. 1). The insulin content of an unknown sample is obtained by reference to the standard curve for the concentration corresponding to the observed B/F ratio.

Preparation of insulin-I^{131} and other technical aspects have been described(11,12).

Most antiserums show quantitative differences in reaction with insulins from different animal species(13). Therefore the present study employed a human antiserum (L.J.) to beef-pork insulin which had previously (13) been shown to react very similarly with beef and pork insulins. Since the glucagon preparations tested are obtained from mixtures of beef and pork pancreatic extracts, the precise contribution of each being unknown, standard curves were prepared with 2 sets of insulin solutions, one containing equal parts crystalline beef insulin and crystalline pork insulin, the other containing pure beef insulin. Since beef insulin reacts slightly more strongly than pork insulin with antiserum L.J., the insulin content of glucagon estimated from the standard beef insulin curve represents a minimum estimate whereas that estimated from the beef-pork insulin standards is probably more realistic.

Both glucagon preparations were analyzed for insulin at concentrations of 20, 30, 40, 50 and 75 µg/ml. In addition, Lot #258-234 B-167-1 was treated with 0.02 M cysteine at pH 8.0 for 12 hours at 37°C to inactivate insulin (14) and was then dialyzed against distilled water for 24 hours to remove the cysteine.

Results. Radioactive scans of the paper strips are shown in Fig. 2 for several of the beef-pork insulin standards and for the glucagon series of Lot #258-234 B-167-1 with and without cysteine treatment. Insulin concentrations determined by reference to the beef-pork insulin and to the beef-insulin standard curves for both glucagon preparations are given in Table I. None of the cysteine-treated mixtures contained any immunologically active insulin. Glucagon Lot #258-234 B-54-2 contained 0.041 ± 0.003% insulin and glucagon Lot #258-234 B-167-1 contained 0.048 ± 0.006% insulin if nearly equal amounts of beef-pork insulin were present. If it is assumed that the preparations contained beef insulin exclusively the results would indicate insulin contents 12% and 10% lower respectively. However, the data themselves provide a better fit to a beef-pork insulin than to a beef insulin standard curve.

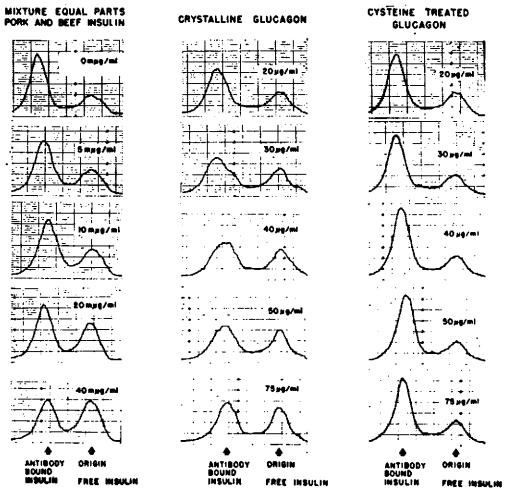

FIG. 2. Radiochromatoelectrophoretograms of mixtures containing trace concentrations of beef insulin-I^{131} and standard solutions of crystalline beef-pork insulin (left), crystalline glucagon (middle) and cysteine treated glucagon (right).

Discussion. Because of possible modifying effects of glucagon on response to insulin in tissues, measurement of the minute insulin contaminant of glucagon by bioassay remains open to some question. Since cysteine has no effect on glucagon itself(8), the absence of any effect of cysteine-treated glucagon preparations on the B/F ratios for insulin-I^{131} provides evidence that glucagon neither augments nor inhibits the reaction of insulin with insulin-binding antibodies; nor would such interference be anticipated in view of the known specificity of antigen-antibody reactions and the demonstration that plasma proteins do not affect the immunoassay of insulin(12).

Although our results are lower than some of those reported previously with tissue assays, the results of Lee *et al.*(8) are lower still, being 0.013% (confidence limits 0.0058%-0.030%) for Lot #258-234 B-54-2. Although these authors could not entirely exclude an effect of glucagon in their system, the cysteine-treated insulin still exhibiting a slight amount of insulin-like activity, the discrepancy in results between the fat pad assay and the immunologic assay cannot be resolved on this basis alone since correction for insulin-like activity of glucagon would have reduced still further the insulin content estimated by the fat pad assay. However, since, in addition, the slope of concentration $C^{14}O_2$ response curve differed for insulin and glucagon preparations, it is possible that glucagon alters the fat pad response to insulin. In any event it appears that the insulin content of the glucagon preparations examined here is significantly less than 0.1%.

Summary and conclusions. 1. The insulin content of each of 2 crystalline glucagon preparations was found to be less than 0.05% by immunochemical assay. 2. Immunologically reactive insulin was not detected in a cysteine treated glucagon preparation.

TABLE I.

Lot	Glucagon conc., μg/ml	Insulin concentration: From beef insulin standard curve mμg/ml	From beef insulin standard curve %	From beef-pork insulin standard curve mμg/ml	From beef-pork insulin standard curve %
258-234 B-54-2	20	5.5	.028	8.4	.042
	30	8.0	.027	12.0	.040
	40	14.0	.037	17.5	.044
	50	22.0	.044	22.0	.044
	75	32.0	.043	28.0	.037
			.036 ± .006*		.041 ± .003
258-234 B-167-1	20	8.0	.040	11.3	.056
	30	9.3	.031	12.8	.043
	40	19.8	.047	21.5	.054
	50	26.0	.052	25.0	.050
	75	35.0	.047	30.0	.040
			.043 ± .007		.048 ± .006
258-234 B-167-1	cysteine treated—no detectable insulin				

* Mean ± S.D.

1. Staub, A., Sinn, L., Behrens, O. K., *J. Biol. Chem.*, 1955, v214, 619.
2. Berthet, J., *Am. J. Med.*, 1959, v26, 703.
3. Randle, P. J., *J. Endocrinol.*, 1958, v17, 396.
4. McNaught, M. L., *ibid.*, 1958, v17, XVI.
5. R-Candela, J. L., R-Candela, R., *Ciba Foundation Colloquia Endocrinol.*, 1956, v9, 194.
6. Snedecor, J. G., DeMeio, R. H., Pincus, I. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 396.
7. Narahara, H. J., Williams, R. H., *J. Biol. Chem.*, 1958, v233, 1034.
8. Lee, H. M., Ellis, R. M., Bromer, W. W., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 4.
9. Berson, S. A., Yalow, R. S., *Advances in Biol. & Med. Physics*, Acad. Press, 1958, v6, 349.
10. Kallee, E., *Z. f. Naturforsch.*, 1952, v7b, 661.
11. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., Newerly K., *J. Clin. Invest.*, 1956, v35, 170.
12. Yalow, R. S., Berson, S. A., *ibid.*, 1960, v39, 1157.
13. Berson, S. A., Yalow, R. S., *ibid.*, 1959, v38, 2017.
14. DuVigneaud, V., Fitch, A., Pekarek, E., Lockwood, W. W., *J. Biol. Chem.*, 1931, v94, 233.

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Metabolic Adaptations in Higher Animals VI. Liver Arginase Activity During Adaptation to High Protein Diet.* (26563)

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Changes in liver arginase activity that represent adaptive responses to dietary changes have been reported from several laboratories. Liver arginase activity per unit of tissue decreases when rats are fed low-protein or protein-free diets(1-4) and increases when they are fed a high-protein diet(4) or are starved (5).

Lightbody and Kleinman(6) first observed that liver arginase activity was responsive to protein intake. They reported that activity per unit of dry liver or per unit of body weight increased with each increase in the dietary level of protein in rats fed on diets containing 6, 25, 60 and 75% protein. Mandelstam and Yudkin(7) also showed that liver arginase activity, per unit of liver or per unit of body weight, in rats was proportional to the amount of protein in their diet and that the response to protein was linear for rats fed on diets containing 20, 40 and 60% of protein. However, Maramatsu and Ashida(8) reported that, although this was true of rats fed on diets containing more than 20% of protein, it did not hold for those fed on diets containing less than 20%.

Little has been reported on the relationship between total arginase activity and amount of urea excreted. A high positive correlation between liver arginase activity and urea excretion was found in an experiment on rats fed for 2 weeks on diets containing 0, 5, 10, 15, 25, 40, and 60% of casein(8), but the results of Rosenthal and Vars(5) who used protein-depleted rats subjected to 2 and 4 days of starvation do not reveal such a correlation. There is also a lack of information about the time-course of the change in arginase activity in response to a change in diet. Mandelstam and Yudkin(7) measured activity at weekly intervals and observed that the response to a change in dietary level of protein reached a maximum within 2 to 3 weeks.

Recently Freedland and Harper(9) reported that a substantial response in liver glucose-6-phosphatase activity occurred within 24 hours after fructose or protein was substituted for "dextrin" in the diet of rats. Therefore, the adaptation of liver arginase during the first few days after feeding rats a high-protein diet has been investigated and the

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